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Abstract: Inflammasomes are immune complexes, which induce an inflammatory response upon sensing of different stress signals. This effect is mainly mediated by activation and secretion of the proinflammatory cytokines prointerleukin(IL)-1 and -18. Here we report that infection of human primary keratinocytes with the double-stranded (ds) DNA viruses Modified Vaccinia Virus Ankara (MVA) or Herpes Simplex Virus Type 1 (HSV-1) induced secretion of mature IL-1 and -18. This secretion was dependent on several inflammasome complexes, however, the AIM2 inflammasome, which is activated by binding of dsDNA, played the most important role. Whereas prestimulation of keratinocytes with interferon (IFN)- moderately increased MVA-induced IL-1 and IL-18 secretion, it was essential for substantial secretion of these cytokines in response to HSV-1 infection. IFN- partially restored HSV-1-suppressed proIL-1 expression and was also required for inflammasome activation. Most importantly, IFN- strongly suppressed virus replication in keratinocytes in vitro and ex vivo, which was independent of inflammasome activation. Our results suggest that, similar to herpesviridae infection in mice, HSV-1 replication in human skin is controlled by a positive feedback loop of keratinocyte-derived IL-1/IL-18 and IFN- expressed by immune cells.

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IFN-γ Primes Keratinocytes for HSV-1–Induced Inflammasome Activation

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Inflammasomes are immune complexes that induce an inflammatory response upon sensing of different stress signals. This effect is mainly mediated by activation and secretion of the proinflammatory cytokines proIL-1β and -18. Here we report that infection of human primary keratinocytes with the double-stranded DNA viruses modified vaccinia virus Ankara (MVA) or herpes simplex virus type 1 (HSV-1)-induced secretion of mature IL-1β and -18. This secretion was dependent on several inflammasome complexes; however, the absent in melanoma 2 (AIM2) inflammasome, which is activated by binding of double-stranded DNA, played the most important role. Whereas prestimulation of keratinocytes with IFN-γ moderately increased MVA-induced IL-1β and IL-18 secretion, it was essential for substantial secretion of these cytokines in response to herpes simplex virus type 1 infection. IFN-γ partially restored HSV-1 suppressed proIL-1β expression and was also required for inflammasome activation. Most importantly, IFN-γ strongly suppressed virus replication in keratinocytes in vitro and ex vivo, which was independent of inflammasome activation. Our results suggest that, similar to *Herpesviridae* infection in mice, HSV-1 replication in human skin is controlled by a positive feedback loop of keratinocyte-derived IL-1/IL-18 and IFN-γ expressed by immune cells.

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INTRODUCTION

Innate immunity is based on germline-encoded receptors, which detect pathogen- and danger-associated molecular patterns. Upon receptor activation, these exogenous and endogenous stress factors induce an inflammatory response, which is required for efficient host defense against pathogens and rapid repair after tissue damage. Inflammasomes comprise a group of cytoplasmic or nuclear complexes, consisting of a central sensor protein such as NACHT, LRR, and PYD domains-containing protein 3 (NLRP3), NLR family CARD domain-containing protein 4 (NLRC4), absent in melanoma 2 (AIM2), or γ-interferon–inducible protein 16 (IFI16), the adaptor apoptosis-associated speck-like protein containing a CARD (ASC), and the effector caspase-1 (Bauernfeind and Hornung, 2013; Schroder and Tschopp, 2010; Strowig et al., 2012). Whereas AIM2 and IFI16 directly bind pathogen-derived DNA and subsequently recruit ASC and caspase-1, the molecular mechanisms underlying detection of danger- and pathogen-associated molecular patterns by other types of inflammasomes are less well understood (Bauernfeind and Hornung, 2013; Dowling and O'Neill, 2012). Upon assembly of inflammasomes, the protease caspase-1 is activated. It then cleaves and thereby activates the proinflammatory cytokines proIL-1β and -18, which induce an inflammatory response after secretion. Inflammation is supported by a lytic and caspase-1–dependent form of cell death termed pyroptosis and by other less well-characterized mechanisms (Miao et al., 2011; Sollberger et al., 2014). In macrophages and dendritic cells, in which inflammasomes have mainly been characterized, IL-1β secretion and inflammasome activation requires a priming step, which induces expression of proIL-1β, NLRP3, and AIM2 (Gross et al., 2011; Hornung and Latz, 2010). However, whether activation of the AIM2 inflammasome in human primary keratinocytes requires priming is not clear (Dombrowski et al., 2011; Kopfnagel et al., 2011) but priming is dispensable for UVB-induced NLRP3 inflammasome activation (Feldmeyer et al., 2007).

Herpes simplex virus type 1 (HSV-1) belongs to the family of *Herpesviridae*, which consists of enveloped viruses with a linear double-stranded DNA genome (Grunewald et al., 2003; van Hal and Dwyer, 2009). It is further classified into the subfamily of the *Alphaherpesvirinae*, whose members are characterized by short replication cycles, destruction of the host cell, and the ability to establish lifelong latency in sensory neurons innervating peripheral tissues (Egan et al., 2013; van Hal and Dwyer, 2009; Whitley, 2011). To do so, HSV-1 must be able to evade the innate immune system, demonstrating its efficient adaptation to humans, which is also reflected in the high seroprevalence of 70% to 80% in the population of developing countries

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Abbreviations: AIM2, absent in melanoma 2; ASC, adaptor apoptosis-associated speck-like protein containing a CARD; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; HSV, herpes simplex virus; ICP, infected cell protein; IFI16, γ-interferon–inducible protein 16; MVA, modified vaccinia virus Ankara; NLRC4, NLR family CARD domain-containing protein 4; NLRP3, NACHT, LRR, and PYD domains-containing protein 3; TNF, tumor necrosis factor; wt, wild type

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(Chayavichitsilp et al., 2009; Paludan et al., 2011; Whitley and Roizman, 2009).

Although HSV-1 infections usually do not cause much harm, they can cause significant morbidity and mortality in immunocompromised individuals by generalization of the infection and brain involvement. Infections usually start at mucocutaneous parts of the skin by targeting keratinocytes (Ellermann-Eriksen, 2005; Paludan et al., 2011).

Productive HSV infection in epithelial cells can be divided into different steps. First, the virion binds to receptors on the surface of the host cell and induces membrane fusion. Viral capsid and tegument proteins such as VP16, which is involved in immediate-early gene transcription, are released into the host cell cytoplasm. Upon transport of the capsid to the nucleus, viral DNA is released into the nucleus. After transcription and translation of viral immediate-early (IE, α) and early (E, β) genes, viral DNA replication is initiated. The early DNA-binding infected cell protein 8 (ICP8) is involved in this process and stimulates late gene transcription. Then transcription and translation of viral late (L, γ) genes coding for capsid and tegument proteins is induced resulting in assembly of viral DNA with the capsid. Finally, newly generated virions are released from the host cell (Taylor et al., 2002).

For an efficient antiviral response, cells must recognize the virus, which is achieved by innate immune receptors of the toll-like receptor family, and mount an immune response. Genomic DNA is most likely the main trigger of HSV-induced innate immune responses (Paludan et al., 2011).

HSV-1 activates the NLRP3 inflammasome in THP-1 cells (Muruve et al., 2008) and the IFI16 and NLRP3 inflammasomes in fibroblasts (Johnson et al., 2013); Kaposi sarcoma-associated herpesvirus activates the IFI16 inflammasome in endothelial cells (Kerur et al., 2011); and mouse cytomegalovirus activates the AIM2 inflammasome in macrophages (Rathinam et al., 2010). Interestingly, the amounts of detected mature IL-1β and IL-18 were extremely low or even undetectable in virus-infected cells (Johnson et al., 2013; Kerur et al., 2011; Milora et al., 2014). For HSV-1 and varicella zoster virus, active immune evasion by targeting of different proteins, which are required for the production of IL-1/-18 activity, has been described (Black et al., 2009; Johnson et al., 2013; Miettinen et al., 2012). Interestingly, IL-1α, which signals through the same receptor as IL-1β, acts as alarmin in HSV-1 infection. IL-1 activity reduces mortality in mice caused by HSV-1, which is most likely mediated by IL-1α, as keratinocytes do not secrete IL-1β upon HSV-1 infection (Milora et al., 2014). Also, other viruses such as poxvirus are able to suppress the production, activation, or activity of IL-1β and -18 (Gram et al., 2012; Lamkanfi and Dixit, 2011; Taxman et al., 2010; Zimmerling et al., 2013).

Because epithelial cells are the major target cell type during primary and recurrent HSV infection, we addressed whether HSV-1 activates inflammasome complexes in human primary keratinocytes. In this study, we found that HSV-1 as well as double-stranded DNA or the double-stranded DNA virus modified vaccinia virus Ankara (MVA) induced maturation of proIL-1β/-18 in human primary keratinocytes, which required expression of proteins of the NLRP3 and AIM2 inflammasomes. However, HSV-1 negatively regulated expression and activation of proIL-1/-18 at different levels, which was counterbalanced by IFN-γ stimulation of keratinocytes. In addition, IFN-γ strongly reduced HSV-1 replication in human primary keratinocytes and in human epidermis ex vivo. Our results suggest that keratinocyte-derived IL-1β or/and IL-18 and IFN-γ expressed by T cells or other cells form a feedback loop important for the control of HSV-1 infections in the skin.

RESULTS

Priming increases DNA-induced AIM2- and NLRP3-dependent IL-1β secretion by keratinocytes

It has recently been demonstrated that transfection of human primary keratinocytes with DNA induces secretion of IL-1β dependent on AIM2 expression (Dombrowski et al., 2011; Kopfnagel et al., 2011). However, whether this DNA-induced IL-1β secretion requires priming with IFN-γ and tumor necrosis factor (TNF)-α is a matter of debate (Dombrowski et al., 2012).

In our hands, transfection of human primary keratinocytes with poly(dA:dT) induced secretion of significant amounts of IL-1β as detected by ELISA (Figure 1a), and this was reproducibly observed with cells from different donors (data not shown). Prestimulation with TNF-α and IFN-γ further increased the levels of IL-1β in supernatants (Figure 1a). Western blotting confirmed the presence of mature IL-1β in supernatants of these cells as well as of processed IL-18 (Figure 1b). In addition, IFN-γ/TNF-α stimulation of keratinocytes induced proIL-1β expression, which most likely contributed to increased amounts of processed and secreted IL-1β.

To confirm that DNA-induced inflammasome activation of keratinocytes depends on AIM2 expression (Dombrowski et al., 2011), we performed siRNA-mediated knockdown experiments in primary keratinocytes (Figure 1c). We already demonstrated that siRNA-mediated suppression of inflammasome protein expression is efficient in keratinocytes (Feldmeyer et al., 2007; Keller et al., 2008; Sollberger et al., 2012). As expected, a knockdown of AIM2 and caspase-1 expression significantly reduced the secretion of mature IL-1β and -18, suggesting that the AIM2 inflammasome is responsible for DNA-induced caspase-1 activation (Figure 1c). Surprisingly, a knockdown of NLRP3 expression also strongly reduced proIL-1β and -18 processing, whereas siRNA, which targets the unrelated vascular endothelial growth factor-A, did not influence cytokine processing.

These experiments demonstrate that in human primary keratinocytes, the AIM2 and NLRP3 inflammasomes are required for efficient caspase-1 activation and subsequent processing of proIL-1β and -18 induced by cytoplasmic DNA. Although priming with TNF-α and IFN-γ increases cytokine maturation, it is not essential.

IFN-γ significantly enhances AIM2-dependent proIL-1β/-18 maturation induced by MVA

MVA, a highly attenuated vaccinia virus generated by extensive passaging in tissue culture from a smallpox vaccine strain (Mayr et al., 1975), is used as a vaccine vector against different infections. The MVA strains MVA-BN and MVA-BN-GFP used in this study are able to transduce human cells but are unable to replicate in these cells (Suter et al., 2009). Recently, it has been demonstrated that MVA activates the NLRP3 inflammasome in THP-1 cells upon infection

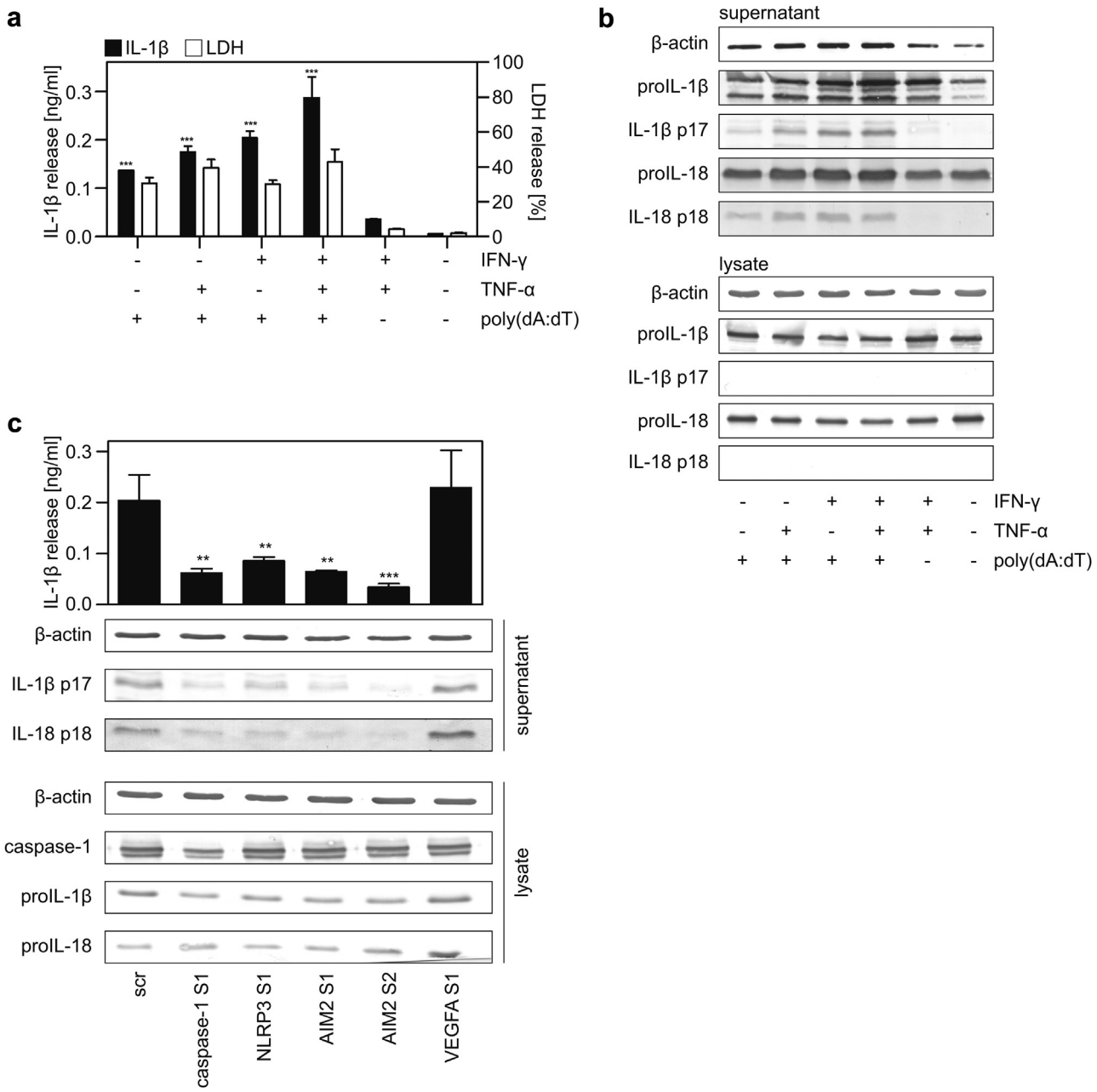

Figure 1. Cytoplasmic DNA activates the AIM2 and NLRP3 inflammasomes in human primary keratinocytes. (**a, b**) Human primary keratinocytes were stimulated with IFN-γ (100 ng/ml), TNF-α (10 ng/ml), or mock-treated, and 24 hours later transfected with poly(dA:dT) (4 μg/ml). Supernatants and cells were harvested after 24 hours and (**a**) analyzed for secretion of IL-1β by ELISA and for cell lysis by lactate dehydrogenase (LDH) assay or (**b**) analyzed for secretion and expression of the indicated proteins by western blot. (**c**) Human primary keratinocytes were transfected with siRNA as indicated (scrambled siRNA served as control) and 3 days later transfected with poly(dA:dT) (4 μg/ml). After 24 hours, supernatants and cells were harvested and analyzed for IL-1β in the supernatant by ELISA or for expression and secretion of the indicated proteins by western blot. Statistics: (**a, c**) Error bars represent the mean ± standard deviation of a representative experiment performed in triplicates. One-way analysis of variance with Dunnett multiple comparison test comparing all values to (**a**, right bar) the mock-treated control or (**c**) scrambled (scr) was performed. $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

(Delaloye et al., 2009). Infection of human primary keratinocytes with MVA induced secretion of IL-1β and consequently inflammasome activation (Figure 2a and b). Prestimulation of keratinocytes with IFN-γ for 24 hours resulted in a significant increase in mature IL-1β in the supernatant (Figure 2a, b, and d). Because it is well known that IFNs play important roles in the defense against viruses, we analyzed the infection rate of keratinocytes with a green fluorescent protein-encoding MVA upon prestimulation with IFN-α, -β, and -γ (Figure 2c). IFN-γ had the strongest inhibitory effect on MVA infection of keratinocytes. In contrast to IFN-γ, type I IFNs reduced proIL-1β and -18 maturation in MVA-infected keratinocytes (Figure 2d), and a role of type I IFNs in the inhibition of proIL-1β production has been described earlier (Guarda et al., 2011). These experiments suggest that inflammasome activation by MVA in keratinocytes is not correlated with the rate of MVA infection. To identify the type of inflammasome complexes that sense MVA

infection of keratinocytes, we knocked down the expression of several inflammasome proteins (Figure 2e). Suppression of expression of caspase-1 and ASC, which are components of all inflammasome complexes, strongly inhibited IL-1β and -18 secretion. Surprisingly, a knockdown of NLRP3 expression, which is required for MVA-induced proIL-1β maturation in THP-1 cells (Delaloye et al., 2009), only slightly reduced secretion of IL-1β by keratinocytes. Although it is difficult to compare the effect of knockdown of different genes, we found a strong dependency of UVB-induced inflammasome activation on NLRP3 expression (Feldmeyer et al., 2007). In agreement with the fact that MVA is a double-stranded DNA virus, a knockdown of AIM2 expression almost completely abolished MVA-induced IL-1β/-18 secretion by keratinocytes (Figure 2e). In addition, IFI16 but not NLRC4 expression contributed to MVA-induced caspase-1 activation in keratinocytes (Figure 2e). The knockdown efficiencies were assessed by quantitative PCR and show strong reduction of gene expression upon siRNA transfection (see Supplementary Figure S1 online). To address how NLRP3 might be activated in this context (Kawamura et al., 2014), we performed experiments with the antioxidant pyrrolidine dithiocarbamate, the calcium chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA) and its cell-permeable version BAPTA tetrakis(acetoxymethyl ester), and the NLRP3 inhibitor glibenclamide (Figure 2f). Upon MVA infection, we observed reduced secretion of IL-1β and IL-18 from cells treated with pyrrolidine dithiocarbamate, BAPTA tetrakis(acetoxymethyl ester), and glibenclamide, suggesting an involvement of reactive oxygen species, intracellular Ca^{2+} levels, and K^+ efflux in inflammasome activation. Because keratinocytes release mature IL-1β/-18 upon MVA infection, it is tempting to speculate that these cytokines might act in an autocrine or paracrine manner on the keratinocytes to protect them from infection. However, such an effect could not be observed in IL-1β/-18–pretreated keratinocytes (Figure 2g). In summary, MVA infection of keratinocytes resulted in activation of the AIM2 inflammasome, but also expression of NLRP3 and IFI16 supported cytokine maturation. Prestimulation of keratinocytes with IFN-γ strongly increased IL-1β/-18 secretion upon MVA infection and reduced the infection rate.

HSV-1 infection activates the AIM2 and NLRP3 inflammasomes in IFN-γ–stimulated human primary keratinocytes

Previous studies demonstrated that HSV-1 infection of keratinocytes does not induce secretion of active IL-1β, although several publications demonstrate inflammasome activation by HSV-1 in other cell types (Johnson et al., 2013; Kerur et al., 2011; Milora et al., 2014). To address this issue, we infected IFN-γ–primed human primary keratinocytes with increasing amounts of HSV-1 and analyzed secretion of IL-1β by ELISA and western blotting (Figure 3a). Surprisingly, we clearly detected IL-1β production upon HSV-1 infection in a dose-dependent manner. However, the amounts of IL-1β were lower than those observed upon poly(dA:dT) transfection or MVA infection (Figures 1 and 2). This was most likely due to inflammasome-dependent caspase-1 activation, because we also detected processing of the caspase-1 substrate proIL-18 upon HSV-1 infection (Figure 3a). HSV-1 infection of keratinocytes induced IL-1β production after 72 hours (Figure 3b). At this time point, the cells detached from the surface of the culture dish, and we detected significant amounts of β-actin in the supernatant, reflecting cell lysis (Figure 3b; other results not shown). In order to examine whether HSV-1 did indeed activate inflammasome complexes, we performed experiments in keratinocytes with siRNA-mediated knockdowns of gene expression (Figure 3c). As expected, expression of caspase-1 and ASC was required for IL-1β and -18 secretion. In addition, a knockdown of AIM2, NLRP3, and IFI-16 expression strongly reduced the maturation of these cytokines, demonstrating that several types of inflammasomes detect HSV-1 in human primary keratinocytes. However, inflammasome activation and secretion of IL-1β and -18 did not influence HSV-1 infection and replication, because we detected the same amounts of viral DNA in infected control keratinocytes and in cells with reduced expression of these critical inflammasome proteins (Figure 3d). These experiments demonstrate that HSV-1 infection induces activation of several types of inflammasomes; however, this results in secretion of only low amounts of IL-1β and -18. Inflammasome activation does not influence HSV-1 replication.

IFN-γ counteracts HSV-1–induced suppression of proIL-1β expression and prevents virus replication

Because IFN-γ prestimulation of keratinocytes strongly enhanced MVA-induced IL-1β and -18 secretion (Figure 2d), we addressed the question of whether IFN-γ primes keratinocytes for HSV-1–induced inflammasome activation. Whereas mock-treated cells secreted only very low amounts of IL-1β and -18 upon infection with HSV-1, IFN-γ priming strongly enhanced the secretion of these cytokines (Figure 4a). In contrast, IFN-α and -β reduced the amount of IL-1β and -18 in the supernatant of HSV-1–infected keratinocytes. All three IFNs reduced HSV-1 replication in keratinocytes, but IFN-γ clearly had the strongest effect (Figure 4b). This effect of IFN-γ was dose dependent (Figure 4c) and was only detected if the cytokine was added more than 24 hours before infection (Figure 4d). To assess whether IFN-γ reduces the expression of HSV-1 entry receptors or coreceptors and thereby affects viral infection, we performed quantitative PCR after IFN-γ priming for nectin (PVRL)-1 and -2, herpes virus entry mediator (HVEM, TNFRSF), myosin 9 (MYH9), paired immunoglobin-like type 2 receptor α (PILRα), macrophage receptor MARCO, and AIM2 as a positive control (Figure 4e). Expression of mRNA of these proteins was either not affected or induced by IFN-γ treatment (see Figure 4e), suggesting that IFN-γ does not limit virus production in keratinocytes through down-regulation of entry receptor expression.

To further determine the physiological relevance of our findings, we tried to infect keratinocytes ex vivo in human skin with HSV-1 (Figure 4f and Supplementary Figure S1b). Human skin was sewed on a culture dish with medium in contact with the lower part and with the outer surface exposed to the air. Then, we generated a full-thickness cut through epidermis and part of the dermis, and HSV-1 was applied to this cut. Keratinocytes, but not cells in the dermis, were infected by HSV-1 as revealed by expression of the

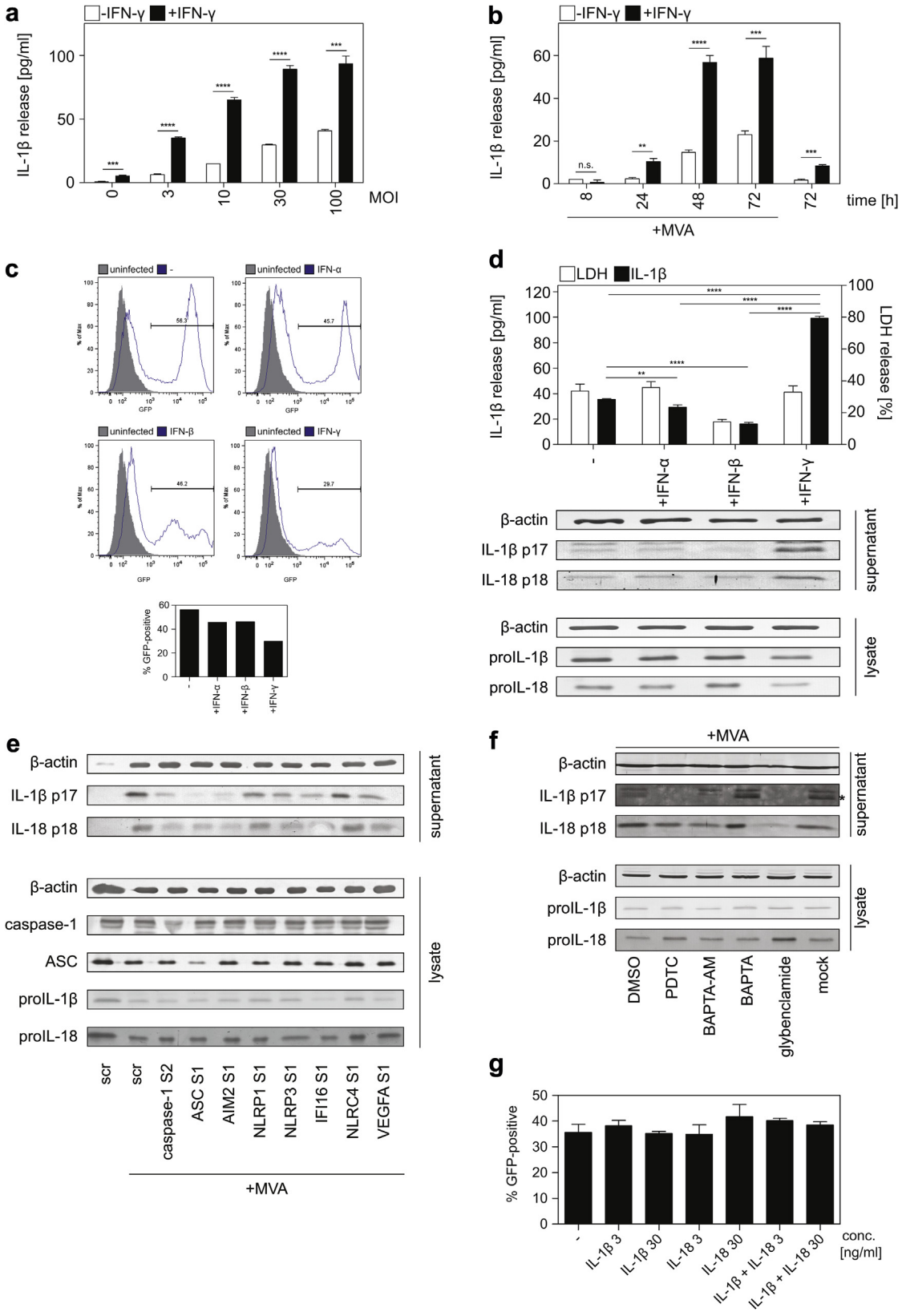

Figure 2. MVA induces AIM2 inflammasome activation in keratinocytes. (**a, b**) Human primary keratinocytes were mock-treated or stimulated with IFN-γ (20 ng/ml). Twenty-four hours later, cells were (**a**) infected with MVA using different MOIs and supernatants were harvested after additional 48 hours or (**b**) infected with MVA (MOI = 2) and harvested at the indicated time points. (**a, b, d**) IL-1β was determined by ELISA. Keratinocytes were left untreated or treated with (**c, d**) IFN-α (1,000 U/ml), IFN-β (1,000 U/ml), or IFN-γ (20 ng/ml) or (**g**) with the indicated concentrations of IL-1β or IL-18 for 24 hours and infected with (**c, g**) a green fluorescent protein (GFP)-encoding MVA or (**d**) with MVA (MOI = 10). (**c, g**) The percentage of GFP-expressing keratinocytes was determined 24 hours later by flow cytometry. (**d**) Supernatants and cells were harvested 48 hours after infection and analyzed for secretion of IL-1β by ELISA and cell lysis by LDH

HSV-1–specific proteins VP16 and ICP8 (Figure 4f and Supplementary Figure S1b). Most importantly, pretreatment of the skin with IFN-γ by topical application strongly reduced the number of HSV-1–infected keratinocytes, as revealed by staining for VP16 and ICP8.

To address the question how IFN-γ primes keratinocytes for IL-1β and IL-18 production, we analyzed the mRNA levels of inflammasome proteins and cytokines in keratinocytes upon HSV-1 infection with and without prestimulation with IFN-γ (Figure 5a and Supplementary Figure S1c). As expected, IFN-γ induced mRNA levels of inflammasome proteins (particularly AIM2) as well as expression of proIL-1β, but not of proIL-18 and IFN-β. Surprisingly, HSV-1 infection of keratinocytes had a similar effect on mRNA expression of inflammasome proteins because the expression of all genes that we analyzed was induced, except expression of proIL-1β and -18, which were downregulated (Figure 5a). IFN-γ stimulation of keratinocytes before infection with HSV-1 increased mRNA levels of several inflammasome proteins compared to infected cells without priming but could not rescue the HSV-1–mediated suppression of proIL-1β and -18 mRNA levels. This result suggests that priming of HSV-1–infected human keratinocytes with IFN-γ does not involve transcriptional regulation of proIL-1β and -18. Western blots revealed that stimulation of keratinocytes with IFN-γ induced protein expression of caspase-1, caspase-4, NLRP3, and proIL-1β (Figure 5b). However, only proIL-1β protein expression was reduced in keratinocytes upon infection with HSV-1, and this reduction was partially rescued by IFN-γ priming.

To address the question whether HSV-1 replication is required for suppression of proIL-1β production, we infected keratinocytes with a replication-deficient HSV-1 (rHSV-1 5dl1.2), which lacks the viral transcription regulator ICP27 (Figure 5c) (McCarthy et al., 1989). Keratinocytes infected with rHSV-1 5dl1.2 produced the same amount of proIL-1β as noninfected control cells, whereas infection with wild-type (wt) HSV-1 reduced proIL-1β protein expression. Consequently, infection of keratinocytes with rHSV-1 5dl1.2 resulted in significantly more secretion of mature IL-1β as infection with wt virus.

Inflammasome activation and secretion of mature IL-1β and IL-18 were also blocked when keratinocytes were treated with other NLRP3 activators, such as UVB irradiation, nigericin, or cycloheximide (Figure 5d). Stimulation with IFN-γ prevented the inhibitory effect of HSV-1 infection. However, neither IL-1β nor IL-18 inhibited HSV-1 replication directly (Figure 5e), nor did HSV-1 infection induce IFN-γ secretion by keratinocytes (results not shown).

DISCUSSION

Here we report that IFN-γ–primed human primary keratinocytes secrete active IL-1β and IL-18 upon HSV-1 infection. Although keratinocytes are infected by HSV-1 at mucocutaneous sites in vivo and represent the primary target of the virus, most reports dealing with HSV-1 infection are based on other cell types. In keratinocytes, HSV-1–induced proIL-1β and -18 maturation depends on the inflammasome proteins AIM2, IFI16, and NLRP3. In contrast, in THP-1 cells expression of NLRP3 is needed for caspase-1 activation, and in fibroblasts IFI16 and NLRP3 are required (Johnson et al., 2013; Muruve et al., 2008). AIM2 is also the most important sensor for MVA-induced caspase-1 activation in keratinocytes, whereas NLRP3 plays the key role in THP1 cells (Delaloye et al., 2009). This suggests that different types of inflammasome complexes sense the same pathogen in different cell types, and that several of these complexes are involved in MVA and HSV-1 detection in keratinocytes.

Compared with MVA, HSV-1 infection of keratinocytes induces secretion of significantly less IL-1β and -18. Interestingly, it has been recently reported that keratinocytes are not able to secrete IL-1β upon HSV-1 infection (Milora et al., 2014). In addition, other reports dealing with inflammasome activation caused by HSV-1 infection or related *Herpesviridae* demonstrate a very low or even undetectable amount of secreted mature IL-1β (Johnson et al., 2013; Kerur et al., 2011; Milora et al., 2014). These results suggest that HSV-1 either is a weak inflammasome activator or actively suppresses the production of IL-1β. In line with the ability of HSV-1 to infect sensory neurons in a latent manner, several immune evasion mechanisms have been demonstrated for *Herpesviridae* (Paludan et al., 2011), including inhibition of the production of IL-1 and IL-18 (Black et al., 2009; Johnson et al., 2013; Miettinen et al., 2012). Our results suggest that HSV-1 suppresses IL-1β production at the posttranscriptional level through reduction of protein expression of the cytokine proform on the one hand and through inhibition of inflammasome activation on the other hand. Interestingly, the HSV-1 immediate-early gene ICP0 encodes an E3 ubiquitin ligase, which is known to counteract immunity (Lanfranca et al., 2014) and to inhibit IFI16/NLRP3 inflammasome activation (Johnson et al., 2013). This raises the possibility that ICP0 is also involved in antagonizing IL-1β/IL-18 production in keratinocytes.

Whereas a function of type I IFNs in antiviral defense is well documented (Stetson and Medzhitov, 2006), our results suggest a particularly important role of IFN-γ in HSV-1–infected human keratinocytes and in human epidermis ex vivo. Prestimulation of keratinocytes with IFN-γ is required for inflammasome activation and secretion of

assay. Expression and secretion of the indicated proteins was determined by western blot. (**e**) Keratinocytes were transfected with siRNA as indicated (scrambled siRNA served as control). Forty-eight hours after siRNA transfection, cells were treated with IFN-γ (20 ng/ml). Forty-eight hours after infection with MVA (MOI = 10) or mock treatment, supernatants and cells were harvested and analyzed for the secretion and expression of the indicated proteins by western blot. (**f**) Keratinocytes were treated with IFN-γ (20 ng/ml) for 24 hours before mock treatment or treatment with dimethylsulfoxid (DMSO; 3 μl/ml), PDTC (500 μM), BAPTA-AM (12.5 μM), BAPTA (25 μM), or glibenclamide (120 μM). Cells were infected with MVA (MOI = 10) 1 hour later. Cells and supernatants were harvested 48 hours after infection and analyzed for expression and secretion of the indicated proteins by western blot. The asterisk marks the relevant band for mature IL-1β. Statistics: (**a, b, d**) Error bars represent the mean ± standard deviation of a representative experiment performed in triplicates. (**a, b**) Unpaired t-test comparing +IFN-γ and −IFN-γ or (**d**) one-way ANOVA with Bonferroni multiple comparison test comparing all values was performed. $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; $^{****}P \leq 0.0001$. BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; BAPTA-AM, BAPTA tetrakis(acetoxymethyl ester); PDTC, pyrrolidine dithiocarbamate.

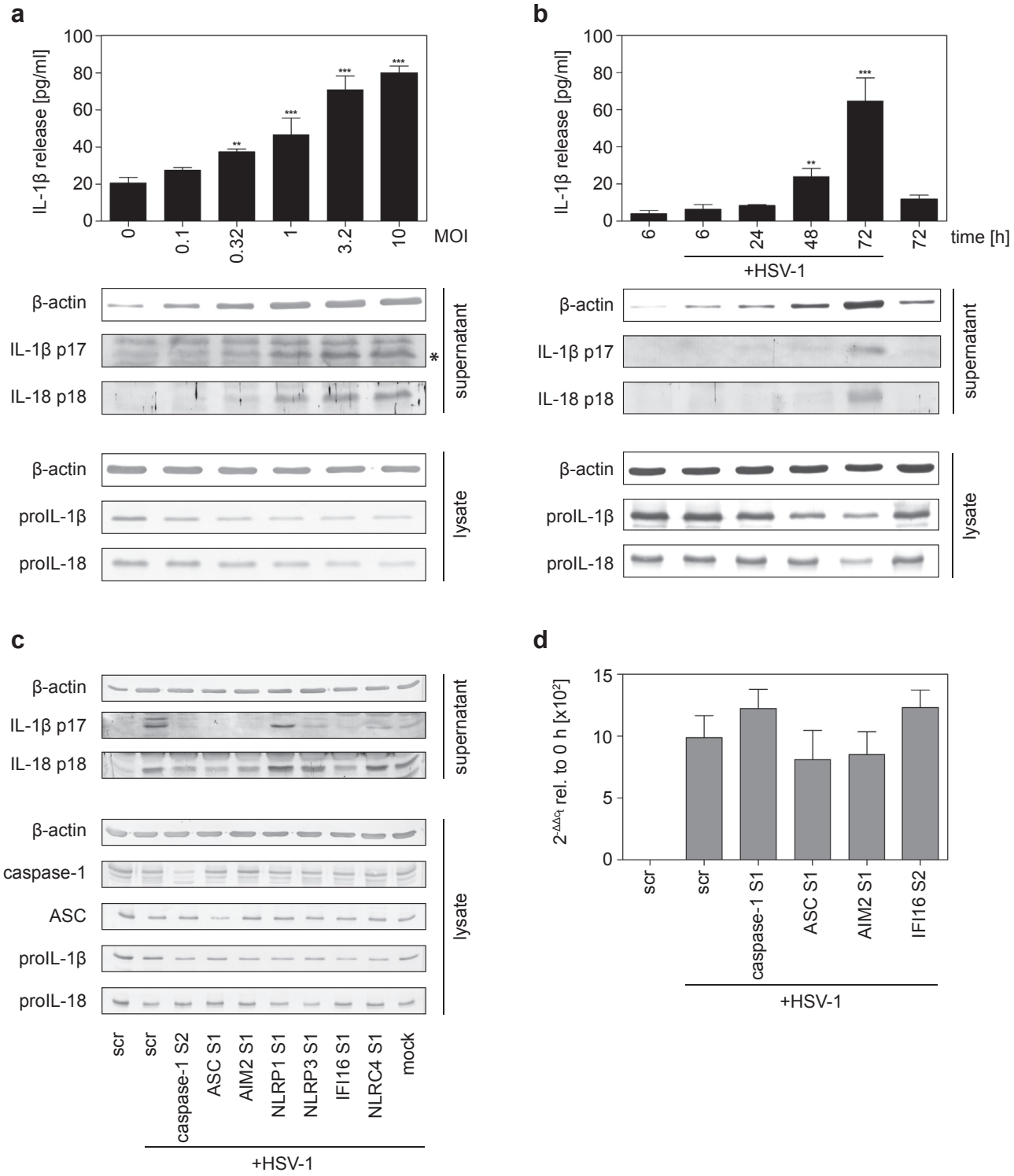

Figure 3. HSV-1 infection of keratinocytes activates different types of inflammasomes. (**a, b**) Human primary keratinocytes were treated with IFN-γ (20 ng/ml) for 24 hours, (**a**) infected with different MOIs of HSV-1 or mock-treated as indicated and harvested after 72 hours or (**b**) infected (MOI = 5) and harvested at different time points as indicated. The asterisk marks the relevant band for mature IL-1β (**a**). IL-1β in the supernatant was determined by ELISA. (**c, d**) Expression and secretion of the indicated proteins were analyzed by western blot. Keratinocytes were transfected with the respective siRNAs (scrambled siRNA served as control) and 24 hours later treated with IFN-γ (20 ng/ml). (**c**) After 24 hours the cells were infected with HSV-1 (MOI = 10), cells and supernatants were harvested 48 hours later and analyzed for the secretion and expression of the indicated proteins by western blot. (**d**) Keratinocytes were treated as described for (**c**) and infected with HSV-1 (MOI = 0.5). Viral replication was quantified by quantitative PCR. Statistics: (**a, b**) Error bars represent the mean ± standard deviation of a representative experiment performed in triplicates. One-way analysis of variance with Dunnett multiple comparison test comparing all values to (**a**) MOI = 0 or (**b**) 6 hours was performed. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$.

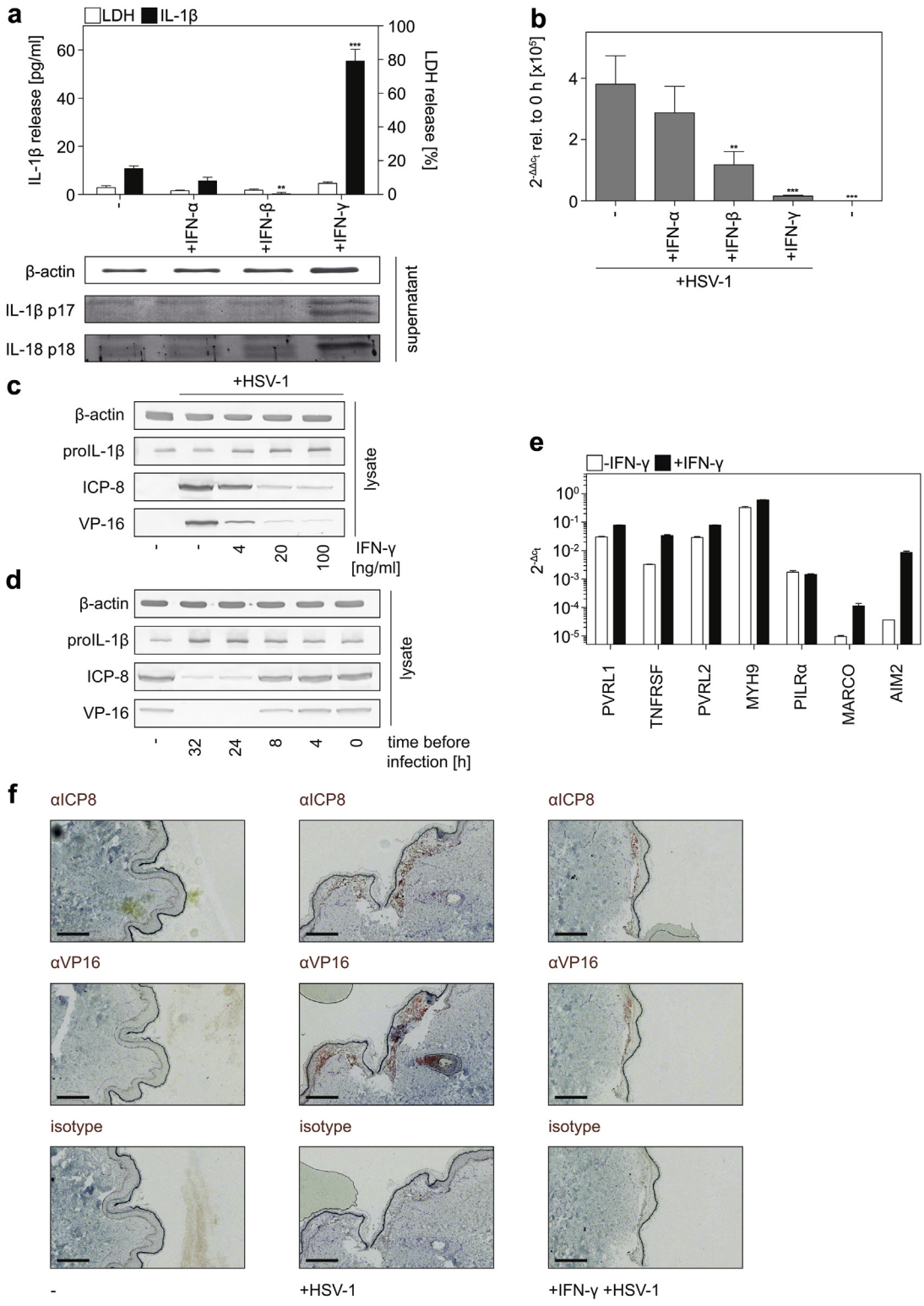

Figure 4. IFN-γ is required for HSV-1–induced IL-1β secretion and inhibits virus replication. (**a, b**) Keratinocytes were left untreated or treated with IFN-α (1,000 U/ml), IFN-β (1,000 U/ml), or IFN-γ (20 ng/ml) and 24 hours later infected with HSV-1 (**a**: MOI = 1; **b**: MOI = 0.5). (**a**) Cells and supernatants were harvested after 72 hours and analyzed for secretion of IL-1β by ELISA and expression and secretion of the indicated proteins by western blot. (**b**) Viral replication was quantified by quantitative PCR 48 hours after infection. (**c, d**) Keratinocytes were treated with IFN-γ (**c**) as indicated or (**d**) with 20 ng/ml, either (**c**) at 24 hours or (**d**) at different time points before infection with HSV-1 (MOI = 1). Western blots were performed for analysis of expression/activation of the indicated proteins. (**e**) Keratinocytes were treated with 20 ng/ml IFN-γ or mock-treated for 24 hours. mRNA levels of the indicated genes were determined by quantitative reverse transcriptase-PCR. (**f**) Human skin was cut with a scalpel ex vivo, and 6.6×10^6 pfu HSV-1 were added with or without prior IFN-γ priming (300 ng applied on cut) for 24 hours. After 5 days, the skin was fixed and paraffin sections were stained with hematoxylin and an antibody against VP16, ICP8, or an isotype control. Bars = 300 μm. Statistics: (**a, b**) Error bars represent the mean ± standard deviation of a representative experiment performed in triplicates. One-way analysis of variance with Dunnett multiple comparison test comparing all values to (**a**) MOI = 0 or (**b**) 6 hours was performed. $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

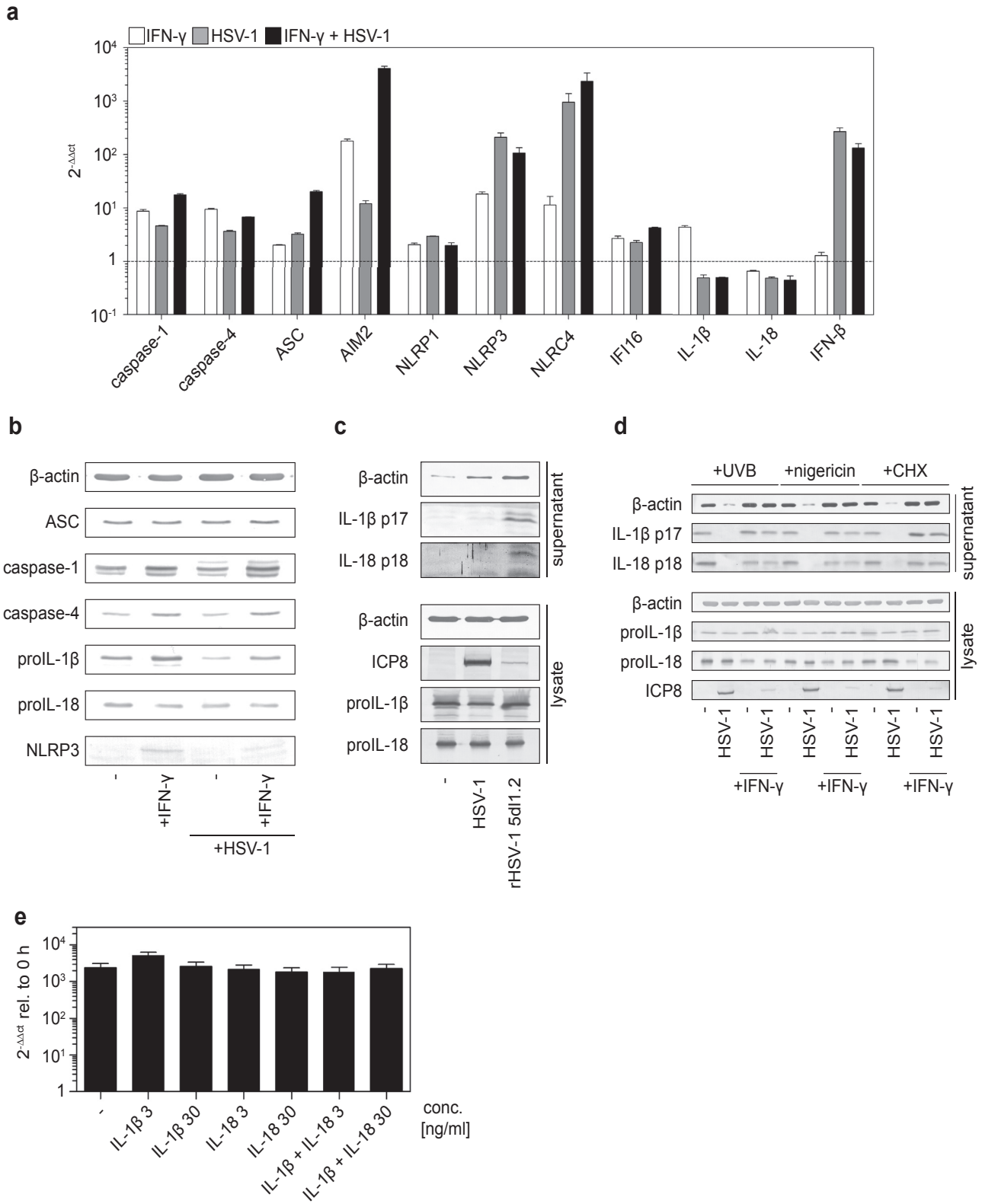

Figure 5. HSV-1 inhibits and IFN-γ enhances production of IL-1β. (**a**–**d**) Keratinocytes were mock-treated or treated with IFN-γ (20 ng/ml) and 24 hours later infected with HSV-1 (**a, b**: MOI = 0.5; **c, d**: MOI = 1) where indicated, (**c**) replication-deficient HSV-1 rHSV-1 5dl1.2, or mock-treated. (**a**) mRNA levels of the indicated genes were determined after 48 hours by quantitative reverse transcriptase-PCR and are shown in relation to the levels before infection. (**b**–**d**) Western blots for detection of the expression/activation of the indicated proteins. (**b**) Cell lysates were harvested 24 hours after infection. (**c**) Lysates and supernatants were harvested 72 hours after infection. (**d**) HSV-1–infected or mock-treated keratinocytes were irradiated after medium change with UVB (86.4 mJ/cm^2) or treated with nigericin (5 μM) or cycloheximide (CHX; 5 μg/ml). Lysates and supernatants were harvested 24 hours later. (**e**) Keratinocytes were mock-treated or treated with the indicated concentrations of IL-1β or IL-18 for 24 hours and infected with HSV-1 (MOI = 1). Viral replication was quantified by quantitative PCR 24 hours after infection.

mature IL-1β and -18. In contrast, IFN-α and particularly IFN-β rather inhibit IL-1β production. Most likely, IFN-γ exerts a general positive priming effect on inflammasome activation in different cell types, which is particularly important for AIM2 expression and therefore AIM2 inflammasome activation. However, comparison of poly(dA:dT)-, MVA-, and HSV-1–induced IL-1β and IL-18 production in keratinocytes reveals a prominent role of IFN-γ for HSV-1–induced inflammasome activation. This is partially because HSV-1 replication suppresses proIL-1β protein expression, which is antagonized by IFN-γ. Most importantly, IFN-γ strongly suppresses virus replication in keratinocytes. However, inflammasome activation is not linked to HSV-1 replication in keratinocytes in vitro.

Recently, it has been reported that IFN-γ inhibits HSV-1 replication in vitro and is also required for the efficient early control of HSV and *Herpesviridae* replication in vivo (Ellermann-Eriksen, 2005; Kim et al., 2013; Rathinam et al., 2010). In IL-18R–deficient mice, IFN-γ expression is strongly reduced after infection with the herpesvirus mouse cytomegalovirus (Pien and Biron, 2000). This demonstrates that IFN-γ expression is at least in part dependent on IL-18 (Ellermann-Eriksen, 2005; Fujioka et al., 1999). In addition to the caspase-1 substrate proIL-18, an important role in defense against HSV was also described for IL-1β (Saha et al., 2010; Sergerie et al., 2007), and IL-1 could be detected in herpetic lesions and keratinocytes of HSV-1–infected human skin (Mikloska et al., 1998). In contrast, MVA induces IL-1β activation but has been shown to alleviate IL-1 activity by expression of a soluble IL-1β receptor (vIL-1βR), which scavenges free active IL-1β. Infection of mice with MVA deleted for the gene encoding vIL-1βR show higher levels of IFN-γ–secreting activated memory $CD8^+$ T cells compared to mice infected with wt MVA (Zimmerling et al., 2013). Interestingly, it has been recently reported that tissue-resident memory $CD8^+$ T cells play an important role in the control of reinfection by HSV-1 through induction of an antiviral state in keratinocytes, most likely mediated by IFN-γ secretion (Ariotti et al., 2014). This IFN-γ secretion may be induced by keratinocytes, as the T-cell response is dependent on viral antigen presentation through infected epithelial cells (Macleod et al., 2014). We speculate that IFN-γ is mostly derived from T cells, because it is reported that cells expressing the natural killer cell marker CD56 can hardly be detected in herpetic lesions, whereas most infiltrating cells are positive for CD3 (Morizane et al., 2005). Then, IFN-γ–stimulated keratinocytes are able to activate caspase-1 upon HSV-1 infection and in turn secrete IL-1 and IL-18. The cytokines directly or indirectly further enhance expression and secretion of IFN-γ, resulting in a positive feedback loop. Such a feedback loop has also been described in mice to be important for control of mouse cytomegalovirus infection (Rathinam et al., 2010). Most importantly, IFN-γ not only enhances IL-1β and IL-18 production, but it also suppresses virus replication in keratinocytes, thereby controlling and limiting virus spread in the epidermis.

In conclusion, our results suggest that IFN-γ may be useful for the treatment of HSV-1 infections in immunocompromised individuals through the induction of IL-1β/IL-18 production and inhibition of virus replication in keratinocytes.

MATERIALS AND METHODS

Viruses

The wt MVA-BN and green fluorescent protein-encoding MVA-mBNbc37#2 were gifts from Bavarian Nordic GmbH (Martinsried, Germany). Vero cells, Vero 2-2 cells (Smith et al., 1992), wt HSV-1 (F strain), and rHSV-1 5dl1.2 (McCarthy et al., 1989) were gifts from the group of C. Fraefel (Zurich, Switzerland).

Cell culture

Isolation and culture of human primary foreskin keratinocytes have been described (Feldmeyer et al., 2007; Keller et al., 2008; Sollberger et al., 2012). Briefly, human primary foreskin keratinocytes were passaged in keratinocyte serum free medium (Gibco BRL, Paisley, Scotland), supplemented with EGF and BPE (Gibco BRL). Cells were seeded for experiments in passage 3. For siRNA-mediated knockdown experiments, cells were seeded in six-well plates at a density of 1×10^5 cells per well. The day after seeding, cells were transfected with siRNA oligonucleotides (10 nM) using 2 μl INTERFERin (Polyplus, Illkirch, France) transfection reagent and left for 48 hours before treatment.

Human biopsies

Human biopsies were collected after obtaining patient informed written consent upon approval from local ethical committees and were conducted according to the principles of the Declaration of Helsinki.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2015.12.022.

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